NMR studies of molecular structure in fruit cuticle polyesters

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Abstract

The cuticle of higher plants functions primarily as a protective barrier for the leaves and fruits, controlling microbial attack as well as the diffusion of water and chemicals from the outside environment. Its major chemical constituents are waxes (for waterproofing) and cutin (a structural support polymer). However, the insolubility of cutin has hampered investigations of its covalent structure and domain architecture, which are viewed as essential for the design of crop protection strategies and the development of improved synthetic waterproofing materials. Recently developed strategies designed to meet these investigative challenges include partial depolymerization using enzymatic or chemical reagents and spectroscopic examination of the intact polyesters in a solvent-swelled form. The soluble oligomers from degradative treatments of lime fruit cutin are composed primarily of the expected 10,16-dihydroxyhexadecanoic and 16-hydroxy-10-oxo-hexadecanoic acids; low-temperature HF treatments also reveal sugar units that are covalently attached to the hydroxyfatty acids. Parallel investigations of solvent-swollen cutin using 2D NMR spectroscopy assisted by magic-angle spinning yield well-resolved spectra that permit detailed comparisons to be made among chemical moieties present in the intact biopolymer, the soluble degradation products, and the unreacted solid residue. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The leaves and fruit of terrestrial plants are protected by a cuticular membrane that mediates wettability, permeability, and microbial resistance. Plant cuticles consist of waxes and cutin, a polyester structural support (Fig. 1). Although it was established two decades ago that cutin’s major monomeric constituents are C\textsubscript{16} hydroxylated fatty acids (Kolattukudy, 1984), the molecular architecture responsible for its support functions has been more elusive because of the biopolymer’s insolubility. Recently, partial degradation of tomato and lime fruit cutin by enzymatic or chemical means has been used to generate soluble oligomeric products that retain essential covalent connectivities from the native plant polyester (Osman et al., 1995; Ray et al., 1998; Ray and Stark, 1998).

The current report reviews the architectural information derived from these cutin oligomers and presents two new approaches to molecular structure determination in fruit cuticles. First, a low-temperature hydrogen fluoride treatment has been used to selectively cleave neutral sugar linkages (Mort and Bauer, 1982; Stark et al., 2000), producing both soluble oligomers and an insoluble residue for spectroscopic analysis. Secondly, the intact polymer has been swollen in organic solvents, generating a suspension from which high-resolution NMR spectra may be obtained using magic-angle spinning (Stark et al., 2000). The results of these studies provide evidence for both hydroxyfatty acid and glycoside building blocks of fruit cutins, and they validate novel spectroscopic strategies for molecular structure determination in cutins, suberins, and related plant materials.
2. Results

2.1. Oligomers from chemical and enzymatic treatments of fruit cutins

In order to augment the identification of molecular groupings in plant polyesters by solid-state $^{13}$C NMR (Zlotnik-Mazor and Stark, 1988; Garbow et al., 1989), a variety of procedures have been developed for partial breakdown of covalent linkages in fruit cutins (Osman et al., 1995; Ray and Stark, 1998; Stark et al., 2000). With each of these approaches, the isolated oligomers retain valuable information regarding the covalent bonding patterns of the native biopolymers.

Using a strategy of partial alkaline hydrolysis, several monomers and a dimer were isolated from the tomato fruit cutin polyester (Osman et al., 1995). The proposed dimer structure contains a primary alcohol ester linkage between two units of 10,16-dihydroxyhexadecanoic acid, the most abundant tomato cutin monomer (Holloway, 1982). Any higher oligomers were deemed undetectable by chemical ionization mass spectrometry (MS). By contrast, enzymatic hydrolysis with *Fusarium solani* cutinase yielded only monomeric products. Nonetheless, this study clearly demonstrated the usefulness of partial degradation procedures in elucidating the essential covalent linkages of protective plant polymers.

Reaction of lime fruit cutin with the enzyme porcine pancreatic lipase, which requires the presence of substrate aggregates and specifically cleaves esters of primary alcohols (Hamosh, 1990), produced a mixture of soluble monomers and oligomers. The pentamer structure 1 was deduced from electron-impact MS experiments tailored for molecular weights up to 1500 amu and with supporting evidence from solution-state NMR spectroscopic analyses (Ray and Stark, 1998). Three of the four building blocks of the pentamer correspond to the principal monomeric fatty acid constituents of Citrus cutins: 10,16-dihydroxyhexadecanoic acid, hexadecanoic acid, and 10-hydroxyhexadecanoic acid (Holloway, 1982; Kolattukudy, 1984; Ray et al., 1995).

Notably, this oligomer structure is assembled solely with linkages between the esters of secondary alcohols, and it lacks the o-hydroxyxoxo fatty acid monomers found abundantly in lime cutin. All four building blocks of the pentamer have molecular structures that render them capable of both polymer chain elongation and cross-link formation within the cutin support structure.

Using the mild chemical deprotecting reagent iodotrimethylsilane, which in organic solution is known to favor hydrolysis of sterically hindered esters of secondary alcohols (Purdy and Kolattukudy, 1975a,b), it was possible to improve the yield of soluble lime cutin fragments from ~5 to 22%. Both monomers and the soluble oligomers 2–4 were identified using two-dimensional multinuclear NMR and liquid secondary-ion mass spectrometry (Ray et al., 1998). The oligomeric materials are each comprised of the two most common Citrus cutin monomers, 16-hydroxy-10-oxo-hexadecanoic acid and 10,16-dihydroxyhexadecanoic acid. Although their covalent architectures are based exclusively on esterification of primary alcohols, the CHI moieties found in the oligomer structures also provide strong evidence for secondary ester linkages in the parent biopolymer.

Finally, we report herein a complementary chemical degradation strategy involving a low-temperature hydrogen fluoride treatment. This type of protocol is known to cleave sugars from glycoproteins (Mort and Lamport,
1977) and to break glycosidic linkages of neutral sugars in polysaccharides preferentially (Mort and Bauer, 1982). Thus, the resulting oligomeric fragments should retain any ester linkages to sugars from cell-wall polysaccharides of the cutin polyester. The low-temperature HF procedure was accompanied by a 23% loss in mass for lime fruit cutin, ensuring that the breakdown products reflect the molecular structure of the intact biopolymer to a significant degree.

A complete structural determination of the soluble products and residues derived from HF treatment is in progress; however, the cutin oligomers examined to date by high-resolution NMR and MS reveal interesting compositional and architectural trends as well as impediments to structural elucidation. The conclusions outlined below are drawn from the following spectroscopic methods: $^1$H and $^{13}$C NMR chemical shifts and integrations; 2D total correlated spectroscopy (TOCSY; Griesinger et al., 1988); gradient-assisted heteronuclear-multiple quantum correlation (gHMQC; Hurd and John, 1991) and gradient-assisted heteronuclear-multiple-bond-correlation (gHMBC; Rinaldi and Keifer, 1994) experiments for through-bond connectivities within each monomeric constituent and between successive monomer units, respectively; and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS; Cotter, 1997) for molecular weights.

First, the most abundant Citrus cutin monomer (16-hydroxy-10-oxo-hexadecanoic acid) is present in all of the oligomers, but the other major lime fruit cutin monomer (10,16-dihydroxyhexadecanoic acid) is absent. The soluble products of low-temperature HF depolymerization also include units of hexadecanoic acid and 16-hydroxyhexadecanoic acid, which were reported previously among the lime cutin monomers and in the pentamer 1 derived from lipase treatment. At least one oligomer structure contains unanticipated sugar moieties, as detailed below. Thus, the significant diversity in monomer composition exhibited by the various oligomers underscores the importance of examining products from several complementary degradative treatments when assembling a molecular picture of the original biopolymer.

Second, the covalent linkages present in the products of low-temperature HF treatment (and the products of other chemical treatments) consist predominantly of primary alcohols esterified to carboxylic acids; only pentamer 1 exhibits secondary ester connectivities. Nevertheless, it would be inappropriate to conclude that lime fruit cutin is lacking in cross-links. Since the reported degradative treatments (Osman et al., 1995; Ray et al., 1995, 1998; Ray and Stark, 1998) are generally incomplete, it is reasonable to assume that heavily cross-linked regions of the insoluble biopolymer are less accessible to the reagents. Moreover, the presence of secondary ester cross links in the intact cutin polymer has been inferred from its extreme insolubility and confirmed by its solid-state $^{13}$C NMR spectra and spin-relaxation times (Zlotnik-Mazori and Stark, 1988; Garbow and Stark, 1990). Supporting evidence for such sequestered regions of lime cutin also comes from the solvent swelling experiments described below.

Third, despite the definitive evidence for ester linkages obtained from long-range through-bond NMR connectivities (Ray et al., 1998; Stark et al., 2000), neither the position of the mid-chain oxo- groups nor the sequence of the monomeric units may be determined from these analyses. As illustrated by oligomer 4, this limitation occurs because the carboxyl, carbonyl, and mid-chain hydroxyl functionalities occur repeatedly and are well separated by spectroscopically degenerate methylene groups. Nevertheless, 10-oxo and/or 10-hydroxy substitution patterns are likely based on the cutin monomer structures found in lime fruit (Ray et al., 1995) and other plant materials (Kolattukudy, 1984). It is also possible, for instance, to verify an alternating arrangement of 16-hydroxy-10-oxo-hexadecanoic acid and 16-
hydroxyhexadecanoic acid units, provided that MS fragmentation patterns or isolated dimers are available (Ray et al., 1998).

Fourth, at least one lime fruit cutin oligomer contains a sugar unit in addition to the hydroxyfatty acid moieties. GC analysis indicates arabinose, glucose, and other unidentified sugar moieties among the chloroform-soluble HF cleavage products, and both the NMR chemical shifts and scalar coupling networks of one purified oligomer are consistent with an unusual 2-deoxyxyranose structure. If the cutin polyester in native Citrus fruit is indeed bound covalently to polysaccharides, then this functionalization of the cell wall may reduce its vulnerability to microbial pathogens, as has been proposed for superized wound periderm (Zucker and Hankin, 1970).

Finally, each of these cutin oligomers poses challenges for MS determination of molecular weights and fragmentation patterns. As noted for the tomato and lime cutin hydrolysates isolated previously (Osman et al., 1995; Ray et al., 1998), volatility will drop off precipitously for compounds larger than dimers. In our hands, liquid secondary-ion mass spectrometry (Aberth et al., 1982) and MALDI-TOF MS (Cotter, 1997) have sometimes failed to give molecular ions for cutin trimers and tetramers; in most cases these techniques will also fail to provide structurally informative fragments. Either derivatization or optimized MS protocols will be needed to confirm and augment the NMR-based structural elucidation of these intriguing plant materials.

2.2. High-resolution NMR of a solvent-swelled lime cutin polymer

Although intact plant cuticular materials may be examined directly by cross polarization magic-angle spinning (CPMAS) solid-state NMR spectroscopy, the resulting $^{13}$C spectral lines are broader than those observed for monomeric and oligomeric fragments in solution (Stark et al., 1989). The corresponding $^1$H NMR spectra display very broad features, even with rapid MAS. These spectral characteristics, which stem from the anisotropic chemical environment at each nucleus and the relative lack of molecular motion, limit the molecular-level structural information available in such amorphous solid materials. Nevertheless, it is possible to regain much of the desired information by swelling cutin in organic solvents (to enhance molecular mobility) and conducting the NMR experiments with MAS (to average chemical shift anisotropy, eliminate dipole-dipole interactions, and minimize magnetic susceptibility line-broadening) (Keifer et al., 1996; Millis et al., 1997; Stark et al., 2000).

Fig. 2 makes a quantitative comparison of MAS $^1$H NMR spectroscopic data for dry and solvated lime cutin, verifying that all of the functional groups observable with vigorous magic-angle spinning are represented in their correct proportions upon swelling of the polyester. The potential of the swelling strategy for molecular structure determination is supported by the high-field MAS $^1$H NMR spectrum of cutin in DMSO (Fig. 3), which exhibits all resonances typical of hydroxyalkanoic acids as well as the oxymethylenes that are diagnostic for esters of primary alcohols (Ray et al., 1998). The improved spectral resolution of this latter spectrum compares favorably with that of a typical lime cutin oligomer (data not shown), demonstrating that near solution-state linewidths may be achieved at slightly elevated temperatures (50°C) and using modest spinning rates (2.5 kHz).

When MAS NMR spectroscopy is combined with standard two-dimensional solution-state spectroscopy, it is possible to elucidate through-bond $^1$H–$^{13}$C interactions and make detailed comparisons between the intact polymer, its soluble degradation products, and its HF-resistant residue. Comparable resolution is observed in the gHMQC contour plots of polymer, residue, and oligomer, again indicating the liquid-like state of the
swelled moieties and showing the promise of the MAS NMR approach.

Fig. 4 displays the 2D MAS-assisted gHMQC data obtained for intact swelled lime cutin (center panel) and for a hydroxyfatty acid-based oligomer derived from low-temperature hydrogen fluoride treatment (top panel). The HMQC ‘fingerprints’ of directly-bonded carbon-hydrogen pairs are somewhat similar for the polymer and oligomer, supporting the presence of numerous distinguishable CH₂O and CH₂ groups in both materials. Surprisingly, no correlations to protonated carbons resonating above 70 ppm are observed in either 2D spectrum, though such resonances have been observed in prior CPMAS ¹³C spectra of the dry solid (Zlotnik-Mazori and Stark, 1988) and attributed to CHOC=O, CHO, aromatic, and doubly bonded functionalities. These signals could be missing from the spectrum of the soluble oligomer because of incomplete breakdown of the parent cutin (see above). Their absence from the gHMQC spectrum of the intact polymer suggests that despite solvent swelling, the combination of motional averaging and 2.5-kHz MAS is insufficient to produce high-resolution NMR spectra for its most heavily cross-linked regions.

As compared with intact cutin, the upfield regions of the oligomer spectrum display cleaner correlation patterns for bulk methylene groups (highlighted by boxes in the upper right-hand corner of each plot) and segments alpha to ester and keto carbonyls (middle boxes). Specific changes in molecular structure are also evident among the oxymethylene groups (lower left boxes). The polymer CH₂OR resonance at (61, 3.4 ppm) disappears in the oligomer, while a new CH₂OH resonance (63, 3.6 ppm) suggests bond cleavage, and the appearance of CH₂CH₂OC=O (60, 4.1 ppm) and CH₂CH₂OC=O (14, 1.2 ppm) signals is indicative of a terminal ethyl ester. An analogous comparison between gHMQC spectra of the polymer and the unreacted residue after HF treatment is also shown in Fig. 4 (center and bottom panels). In this latter case the NMR fingerprints are almost identical, exhibiting differences only among the intensities of selected bulk methylene and terminal methyl cross peaks.

3. Discussion

A detailed understanding of the molecular architecture of plant cutins can point the way toward the design of improved crop protection protocols and robust synthetic waterproofing materials. Although the intractability of these and related plant biopolymers has slowed their structural characterization, it is possible to overcome many of these difficulties using two complementary strategies: partial depolymerization to produce soluble oligomers and high-resolution spectroscopy of the intact cuticular materials.

Soluble oligomers isolated from a variety of chemical and enzymatic degradation treatments on fruit cutins have revealed many of the essential primary and secondary ester linkages present in the native cuticular polymer. Although the through-bond correlations mapped out in 2D NMR spectra provide invaluable structural clues to the identity of these oligomeric materials, important information regarding their molecular weights and positional isomerism remains challenging to obtain routinely by MS methods. Taken together, the monomeric compositions of the oligomers are in reasonable accord with the expectations of exhaustive depolymerization studies, but no single protocol offers a reliable
molecular view of the biopolymer. Esters of secondary alcohols may be underrepresented among the soluble cutin oligomers, because the most heavily cross-linked regions of the biopolymer could be inaccessible to the depolymerization reagents. The possible presence of a novel glycoside oligomer also suggests a role for cutin in blocking access of pathogens or cell-wall degrading agents.

Insoluble cutin polymers and unreacted residues have been rendered amenable to high-resolution NMR structural analysis by swelling them in organic solvents and applying magic-angle spinning techniques. Although this hybrid (semi-solid) NMR strategy must be applied with caution to the cutin regions of heaviest cross-linking, it can provide well-resolved spectral data from both one- and two-dimensional NMR experiments. Thus, it has been possible to compare chemical moieties of the intact cutin polymer to both soluble oligomers and insoluble residues and to delineate some of the corresponding molecular transformations.

The results presented above for lime fruit cutin illustrate the feasibility of obtaining structural information on both oligomeric constituents and intact polymers. Such chemical and spectroscopic approaches also offer promise for detailed investigations of molecular architecture in a wide variety of complex plant materials, including suberins, lignins, and cell-wall polysaccharides.

4. Experimental

4.1. Isolation of lime fruit cutin

Fruit cutin was isolated from the skin of limes (*Citrus aurantifolia*) using a three-step protocol (Pacchiano et al., 1993): (1) peeling and separation of the cuticle by treatment with pectinase; (2) enzymatic digestion of cell-wall polysaccharides with successive cellulase, pectinase, and hemicellulase treatments; (3) exhaustive dewaxing using successive Soxhlet extraction with methanol, methylene chloride, and tetrahydrofuran. All reagents were obtained from Sigma or Aldrich Chemical Companies. In a typical preparation, 200 limes yielded 5.0 g of dry cutin.

4.2. Generation of lime cutin oligomers

In order to produce oligomeric fragments, 502 mg of powdered lime fruit cutin was reacted for 0.5 h with anhydrous HF at 0°C and quenched by diethyl ether in liquid nitrogen (Mort, 1983). After evaporating the ether-HF mixture (120 mg) to dryness, the reaction products were extracted to yield 22 mg of water solubles and 95 mg of chloroform solubles. The unreacted solid residue was reserved for analysis by magic-angle spinning NMR. The chloroform fraction was dried and then extracted with acetonitrile.

Purification of the acetonitrile extract was achieved using reversed-phase HPLC conducted on a Hewlett-Packard Model 1100 instrument equipped with a quaternary solvent delivery system, UV detector, and diode array detector. Separations were performed with 5 μm analytical and semi-preparative C18 columns, using UV detection at 210 nm, a 1-ml/min flow rate, and gradient elution (acetonitrile/H2O/THF, varied from 75:20:5 to 95:0:5 during a period of 20 min). Isolated compounds (typical yield, 0.5 mg) were checked for purity by re-injection in two solvent systems.

4.3. Gas chromatography

Sugars were analyzed as their trimethylsilyl methyl glycosides using slight changes to the procedure of Chaplin, (1982) as previously modified by Komalavilas and Mort (Maness and Mort, 1989). After at least 3 h of methanalysis at 80°C using 1.5 M HCl in dry methanol containing 10% methyl acetate as a water scavenger, a few drops of t-butanol were added and the sample was evaporated to dryness. A 25–40 μl aliquot of a 1.3 v/v mixture of Tri-Sil concentrate (Pierce Chemical Co.): pyridine was added. After 15 min the sample was evaporated just to dryness and then dissolved in 100 μl isooctane. A 1-μl aliquot was injected via a cold on-column injector (J&W Scientific) fitted to a Varian 3300 chromatograph with a flame ionization detector. The column was a 30 m×0.25 mm I.D., 0.25-μm film DB-1 capillary from J&W Scientific with a constant head pressure of 12 psi helium for the carrier gas. During the injection the oven was held at 105°C. After a 1-min hold the temperature was raised to 160°C at a rate of 10°C/min. After a 4-min hold the temperature was then raised to 200°C at 2°C/min for the separation. The temperature was raised to 240°C after each separation to purge the column in preparation for subsequent injections.

4.4. Solution-state NMR spectroscopy

NMR spectra were acquired on a Varian UNITYINOVA spectrometer operating at 1H and 13C frequencies of 599.95 and 150.87 MHz, respectively. Cutin oligomer samples were dissolved in CDCl3 to provide a field-frequency lock signal and contained 1% tetramethylsilane (TMS) to provide an internal chemical shift standard (Aldrich). One dimensional 1H spectra were acquired using a Varian nanoprobe (see below). Typical experimental conditions included 16 repetitions, 2.0 s between successive acquisitions, 26,240 time-domain points, and a spectral width of 7200 Hz. Data processing and 1H peak integration were done using VNMR software; 13C chemical shift predictions were made using database software from Advanced Chemistry Development (Toronto, Canada).

Several two-dimensional NMR experiments were used to establish through-bond connectivities within and
between monomer units of the oligomer structures. 

$^1$H–$^1$H total correlation spectroscopy (‘clean’ TOCSY) (Griesinger et al., 1988) was conducted with an effective spin-lock field of 6.25 kHz and a mixing period of 70 ms in order to allow magnetization transfer throughout each scalar-coupled spin system. Heteronuclear multiple quantum correlation ($^{13}$C HMQC) spectroscopy (Muller, 1979) was used to identify bonded proton-carbon pairs using a polarization transfer time corresponding to $J_{CH} = 140$ Hz. Heteronuclear multiple bond correlation ($^{13}$C HMBC) spectroscopy (Bax and Summers, 1986) was employed to delineate long-range proton-carbon bonded interactions, using a polarization transfer time corresponding to $J_{CH} = 10$ Hz. In proton-detected HMQC experiments, the GARP sequence (Shaka et al., 1985) was used for $^{13}$C decoupling, and pulsed field gradients (Hurd and John, 1991; Rinaldi and Keifer, 1994) were used for coherence selection and to minimize spectral artifacts. The HMQC data were typically acquired with spectral widths of 7200 Hz for $^1$H and 27,150 Hz for $^{13}$C, a relaxation delay of 1.0 s between transients, and an array of 2 K $\times$ 256 points, zero-filled to 2K $\times$1K points. The HMBC data were typically acquired with spectral widths of 7200 Hz for $^1$H and 36,200 Hz for $^{13}$C, a relaxation delay of 1.0 s between transients, and an array of 2K $\times$ 256 points, zero-filled to 2K $\times$1K points.

**Pentamer 1:** see Ray and Stark (1998). **Dimer 2, dimer 3,** tetramer **4:** see Ray et al. (1998).

### 4.5. Magic-angle spinning NMR spectroscopy

MAS $^1$H spectra of swelled cutin samples were acquired either at 600 MHz (as described above) or on a Varian UNITY plus spectrometer operating at a $^1$H frequency of 300.001 MHz. The former experiments were performed with a 4-mm Varian inverse-detection HX nanoprobe equipped with pulsed field gradients, using 1–2 mg of solid sample and magic-angle spinning typically regulated at 2.200±0.001 kHz. The latter experiments were performed on 30-mg samples in a Doty 5-mm XC5 probe spinning at 8.000±0.005 kHz. Spectra were obtained at either room temperature or 50°C, as noted. $^1$H chemical shifts were referenced to DMSO at 2.50 ppm or CDCl$_3$ at 7.24 ppm, and $^{13}$C chemical shifts were referenced to DMSO at 39.51 ppm.

### 4.6. Mass spectrometry

Electron-impact mass spectrometry was carried out at 70 eV using a Finnigan MAT-90 double focusing spectrometer and an accelerating potential of 5 keV, as described previously (Ray and Stark, 1998). Liquid secondary ion mass spectrometry was conducted with a VG ZAB-T high-resolution spectrometer equipped with a 30 kV cesium gun ionizing source, accelerating potential of 8 kV, and dithiothreitol/dithioerythritol matrix (Ray et al., 1998). Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) was carried out using a Perseptive Biosystems Voyager instrument in the positive-ion mode. The matrices used in these experiments were trihydroxacetophenone or indolacrylate (2 mg/ml) in acetone. Data were acquired by signal averaging of 40–70 scans, each obtained with an accelerating voltage of 25 kV and a low-mass gate of 300 amu.

**Pentamer 1:** see Ray and Stark (1998). **Dimer 2, dimer 3,** tetramer **4:** see Ray et al. (1998). Oligomer from low-temperature HF treatment: $m/z$ (M + H)$^+$ 853.

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